ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2014, *59*(11-12), 1047-1057

Dedicated to the memory of
Professor Candin Liteanu on his 100th anniversary

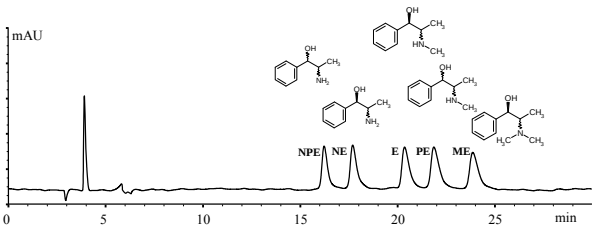
ON THE HPLC/MS-MS ASSAY OF EPHEDRINES IN URINE: AN EXPERIMENTAL APPRAISAL

Elena Emilia IORGULESCU,[a] Florin ALBU,[a,b] Florentin TACHE[a] and Andrei MEDVEDOVICI[a,*]

[a] University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, # 90-92 Panduri Ave., Bucharest-050663, Roumania
[b] Analytical Application Laboratory Agilrom, # 40S Th. Pallady Ave., Bucharest-032266, Roumania

Received September 28, 2014

Chromatographic separation of ephedrines faces the imperative discrimination among the diastereoisomeric pairs ephedrine/ pseudoephedrine and norephedrine/norpseudoephedrine, respectively. Additionally, specific elution mechanisms and/or conditions are required to adequately control the peak symmetry. A discussion about the necessary experimental conditions to fulfil such goals is presented. Three alternatives were selected for the assay at sub-ppm level of ephedrines in urine through HPLC/MS-MS: i) ion pair reversed phase liquid chromatography with a perfluorinated ion pair agent; ii) reversed phase liquid chromatography on a phenyl modified silicagel as a stationary phase; iii) on-line solid phase extraction and reversed phase liquid chromatography under alkaline elution conditions. These alternatives were comparatively discussed with respect to their quality specifications.



INTRODUCTION

Ephedrines are commonly used as stimulants, decongestants, and appetite suppressants. Ephedrines belong to the alkaloids class, acting through the increase of noradrenaline on adrenergic receptors. Due to their increased ability to cross the blood brain barrier, they are acting as central nervous system stimulants, similar to amphetamines. Ephedrines are usually isolated from plants (Ephedraceae family). Analytical applications involving ephedrines refers to their assay in materials of natural origins,[1,2] in pharmaceutical formulations or dietary supplements,[3-5] forensic studies[6-9] (*i.e.* assay in human plasma, urine, or hair for anti-doping control) and monitoring in waste water.[10] The congeners of this class are (see Fig. 1): ephedrine (E), pseudoephedrine (PE), norephedrine (NE), norpseudoephedrine (NPE), and N-methyl ephedrine (ME). The pairs E/PE and NE/NPE are diastereoisomers. Consequently, their chromatographic reciprocal resolution is expressly needed, as long as, even mass spectrometry (MS), with its intrinsic tunable selectivity, is unable to discriminate among them. Certainly, relating to ephedrines, the chiral discrimination on adequate chiral selectors represents another major topic, often discussed in the literature.[11-13]

* Corresponding author: avmedved@yahoo.com

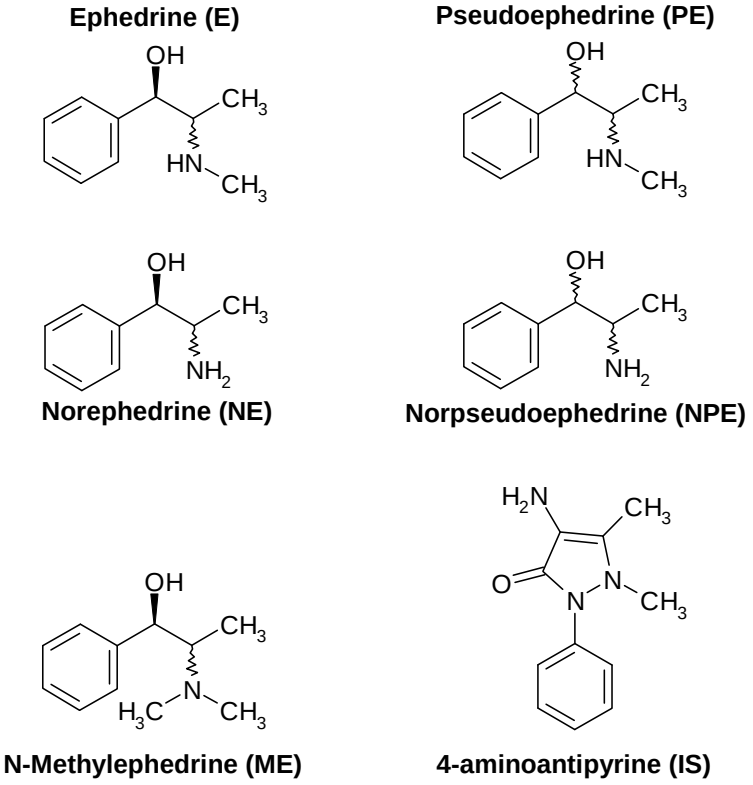


Fig. 1 – The chemical structures of the studied compounds.

Although simple with respect to their chemical structures (basically, ephedrines are derived from phenyl ethylamine), the chromatographic separation of ephedrines is not really an easy task. Due to their alkaline character (corresponding pK_a values are placed in the 9-10 interval), the major problem arising during the chromatographic elution refers to the control of the peak symmetry. The reversed phase (RP) separation mechanism on apolar alkyl modified silicas was always preferred for the separation and quantitation of ephedrines,[14] despite some evident limitations related to peak symmetry and selectivity within the critical pairs. Other attempts deal with the RP separation of ephedrines on perfluorinated stationary phases,[15] under ion pair-reversed phase (IP-RP), or normal phase (NP)[16] and hydrophilic interaction liquid chromatography (HILIC).[17,18] Other analytical techniques, such as capillary zone electrophoresis (CZE),[19] capillary electrochromatography (CEC),[20] micellar electrokinetic chromatography (MECK),[21,22] head-space gas chromatography (HS-GC),[23] ion mobility mass spectrometry (IMMS),[24] isotope ratio mass spectrometry (IRMS),[25] diffuse reflectance infrared Fourier transform spectrometry (DRIFTS)[26] and potentiometric sensors[27] were involved in the analysis of ephedrines. Derivatization was often used to improve sensitivity, while various preparation and pre-concentration techniques were applied to isolate ephedrines in different matrices.[19,20-22,28-30]

The present work represents a realistic evaluation of the experimental alternatives existing for isolation of ephedrines from urine (as an analytical tool useful to the antidoping control), their chromatographic separation and detection through tandem mass spectrometry (MS-MS). After development and optimization, three alternatives were finally selected for the assay at sub-ppm level of ephedrines in urine through HPLC/MS-MS: i) ion pair reversed phase liquid chromatography with a perfluorinated ion pair agent; ii) reversed phase liquid chromatography on a phenyl modified silicagel as a stationary phase; iii) on-line solid phase extraction and reversed phase liquid chromatography under alkaline elution conditions. These alternatives were comparatively discussed with respect to their quality specifications.

EXPERIMENTAL

1. Instrumentation

Experiments were performed with an Agilent 1200 series LC/MSD (Agilent Technologies) system consisting of the

following modules: degasser (G1379A), binary pump (G1312A), automated injector (G1329A with the corresponding thermostat G1330B, respectively), column thermostat (G1316A), ESI standard interface (G1948B), diode array detector (G1315B) and triple quadrupole mass spectrometric detector (G2571A) having an ES ion source (G1948B). System control, data acquisition and interpretation were made with the Agilent MassHunter software version B 01.00 incorporating both qualitative and quantitative packages or by means of the Chemstation software rev. B01.03. (Agilent Technologies). The system was operationally qualified before use. The vortex system was model Multi Reax from Heidolph (Schwabach, Germany) and the thermostated centrifuge was model Universal 320R from Hettich (Tuttlingen, Germany). During method development, diode array detection (DAD) was used, through monitoring at the analytical wavelength of 220 nm. When MS-MS was used as detection system, the ESI parameters were: working mode - positive; nebulising gas pressure - 60 psi; drying gas - 10 L/min; drying gas temperature - 350 °C; capillary voltage - 3500 V. For NPE the collision energy (CID) was 25 V, while for the other compounds it was set to 15 V. The following mass transitions were used to detect analytes (the first one represents the quantifier, the second one the qualifier): for E/PE - m/z 166 to 148 Da; m/z 166 to 117 Da; for NE/NPE - m/z 134 to 91 Da; m/z 134 to 115 Da; for ME - m/z 180 to 162 Da; m/z 180 to 147 Da; for the internal standard (IS, 4-aminoantipyrine) - m/z 204 to 56 Da; m/z 204 to 94 Da. For quantitative purposes, the tandem MS detector was used in the multiple reaction monitoring (MRM) mode.

2. Materials

Acetonitrile, *i*-propanol and methanol HPLC gradient grade from Merck (Darmstadt, Germany) were used during experiments. Water for chromatography (resistivity of minimum 18.2 MΩ and total residual organic carbon - TOC - of maximum 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument. Acetic acid, formic acid, phosphoric acid, ammonium formate and acetate were extra pure grade from Merck. The heptafluorobutyric acid (HFBA) and triethylamine (TEA) were analytical grade quality (> 99.0% and > 99.5%, respectively) from Sigma-Aldrich, as well as ionic liquids used during experiments and the 4-aminoantipyrine - IS (spectrometric grade). Ion pair agents were IPC grade from Merck. Ephedrines standards (> 99.0% purity) were offered by the Laboratory for Doping Control (Roumanian Anti-Doping Agency) which is kindly acknowledged.

3. Chromatographic conditions

The following chromatographic columns were used: **Column A**/ Zorbax Eclipse XDB C18, 150 mm L × 4.6 mm i.d. × 5 μm d.p. (Agilent Technologies); **Column B**/ Zorbax CN, 150 mm L × 4.6 mm i.d. × 5 μm d.p. (Agilent Technologies); **Column C**/ LiChrosorb HPLC C18, 250 mm L × 4.6 mm i.d. × 5 μm d.p. (Merck); **Column D**/ Betasil Phenyl, 250 mm L × 4.6 mm i.d. × 5 μm d.p. (Thermo Electron Corporation); **Column E**/ Luna C18(2), 250 mm L × 4.6 mm i.d. × 5 μm d.p. (Phenomenex); **Column F**/ Purosphere Star C18 end-capped, 250 mm L × 4.0 mm i.d. × 5 μm d.p. (Merck). All columns were exploited under isocratic elution conditions, at flow rates of 0.8 mL/min (when MS-MS detection was used), or 1.0 mL/min (during method development, with UV detection). Specific mobile phase compositions are further described in the text. The chromatographic column was thermostated at 25 °C.

Three alternatives were considered optimal and were subjected to validation. **Method 1** was optimized under IP retention mechanism on **Column E**, using, as mobile phase constituents, an aqueous 0.15% HFBA (v/v) solution and an organic mixture of methanol/*i*-propanol 4/1 (v/v) as organic modifier, in a volumetric ratio of 7/3 (v/v). **Method 2** was optimized under RP retention mechanism (modulated by the π-π interactions produced over the phenyl groups) on **Column D**, using, as mobile phase constituents, an aqueous 0.1% formic acid (v/v) solution and methanol as organic modifier, in a volumetric ratio of 78/22 (v/v). **Method 3** was optimized under RP retention mechanism (under alkaline elution conditions) on **Column F**, using as constituents of the mobile phase an aqueous 0.015% formic acid solution brought to pH=10.5 with TEA and methanol as organic modifier, in a volumetric ratio of 55/45 (v/v) and a flow rate of 1 mL/min.

For the on-line SPE/RPLC set-up used in **Method 3**, the cartridge used for isolation of the target compounds was a vinyl pyrrolidone/styrene divinyl benzene copolymer (Oasis® HLB, 20 mm L × 3 mm i.d. × 5 μm d.p. from Waters). The carrier solvent through the Oasis cartridge was an aqueous 0.015% formic acid solution brought at pH=10.5 with TEA at 1 mL/min flow rate. The cartridge was rinsed with the mobile phase 5 min before valve switching, meaning the backflush desorption of the analytes in the analytical column.

4. Sample preparation

Sample preparation procedures differ according to the chromatographic method being used. For **Method 1**, 1 mL of urine was vortexed (during 5 min, and 2000 rpm rotation speed) with 0.2 mL of IS solution (25 μg/mL) in methanol. After centrifugation for 5 min at 25 °C and 9000×g, 0.5 mL from the supernatant were taken and vortexed with 0.5 mL of the aqueous component of the mobile phase and 20 μL of concentrated formic acid. After mixing for 5 min at 2000 rpm rotation speed, the solution was transferred in a vial. The injected volume was 5 μL.

For **Method 2**, 1 mL of urine was vortexed (during 5 min, and 2000 rpm rotation speed) with 50 μL of the IS (100 μg/mL) solution in methanol. After centrifugation for 5 min at 25 °C and 9000×g, an aliquot from the supernatant was transferred to the injection vial. The injected volume was 5 μL.

For **Method 3**, 1 mL of urine was vortexed (during 5 min, and 2000 rpm rotation speed) with 50 μL of the IS (100 μg/mL) solution of in methanol and 50 μL of 10% TEA (v/v) solution in methanol. After centrifugation for 5 min at 25 °C and 9000×g, an aliquot from the supernatant was transferred to the injection vial. The injected volume on the Oasis HLB cartridge column was 100 μL. The on-line SPE/RPLC set-up is given in Fig. 2.

RESULTS AND DISCUSSION

1. Method development and optimization

Experimental approaches made for separation of ephedrines on octadecyl silicagel stationary phases are illustrated in Fig. 3. One can observe from Fig. 3A that the RP mechanism running on a strongly end-capped stationary phase may ensure enough selectivity of the ephedrine separation

when 100% aqueous mobile phase is used. However, peak symmetry is very poor, and readily cancels the selectivity. From Fig. 3C on can observe that a less end-capped octadecyl stationary phase looses the ability to discriminate against the critical pairs NE/NPE and E/PE. Peak symmetry remains poor, although an ammonium salt was added to the mobile phase to reduce the activity of the residual silanols. Application of an IP-RP mechanism solves, as expected, the issue of peak symmetry (see Fig. 3B). The resulting chromatographic selectivity is however low: NE and PE coelute, while NPE, E and ME are not baseline separated. It is interesting to note that even such a doubtful chromatographic result may be eventually compensated by the selectivity of a tandem MS spectrometer used as detection system. Unfortunately, MS detection does not operate due to the presence in the mobile phase of low volatile ion pairing agents (particularly sodium hexane sulphonate). Improved selectivity and fair peak symmetry may be observed when using a fully aqueous mobile phase with addition of an ionic liquid (butylmethylimmidazolium triflate), also described in the literature.[31] The organic cation of the ionic liquid is retained by means of its alkyl moiety to the end of the octadecyl chains of the stationary phase, creating the possibility of a repulsive electrostatic interaction with the positively charged analyte, resulting from protonation in the elution conditions. The ionic liquid also successfully compensate the negative charge of the ionized silanols and potentially transform such a site in a positively charged one (one ionized silanol and 2 cations of the ionic liquid, reciprocally interacting through their alkyl moieties). Although the chromatographic results being obtained are remarkable from both points of view, selectivity and peak symmetry, respectively, the use of an additive with low volatility in the mobile phase (the ionic liquid) cancels the possibility of using MS as detection system.

It seemed obvious that ephedrines separation under a "pure" RP mechanism is difficult to obtain. We thought that if additional interactions are involved (*i.e.* π-π interactions) better results would be achieved in terms of selectivity and peak symmetry. Trials made for separation of ephedrines on a cyanopropyl chemically modified silicagel are illustrated in Fig. 4. If acetonitrile is used as organic modifier in the mobile phase (see Fig. 4A) only group separation was achieved (NE/NPE and E/PE pairs coelute). Shifting from acetonitrile to methanol as organic modifier in the mobile phase leads to a sensible increase of the chromatographic selectivity (Fig. 4C), the critical pairs NE/NPE and E/PE being resolved almost at baseline. Severe peak tailing affects the last eluting compounds. Peak symmetry problem remains unsolved through transition versus an IP mechanism on the cyanopropyl stationary phase. As one can observe from Figure 4D, the use of sodium pentane sulphonate does not add selectivity within the two critical pairs and does not avoid peak tailing. Introduction of a volatile ion pairing reagent, more precisely a perfluorinated one (HFBA), as illustrated in Fig. 4B, improves selectivity within the critical pairs but does not necessarily increase peak symmetry.

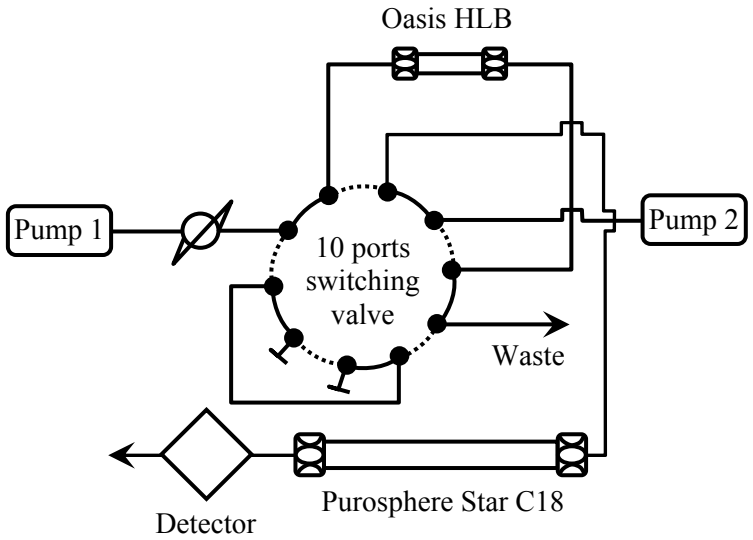


Fig. 2 – The experimental set-up for the on-line SPE/RPLC analysis of ephedrines in urine. Details are given in the Experimental section.

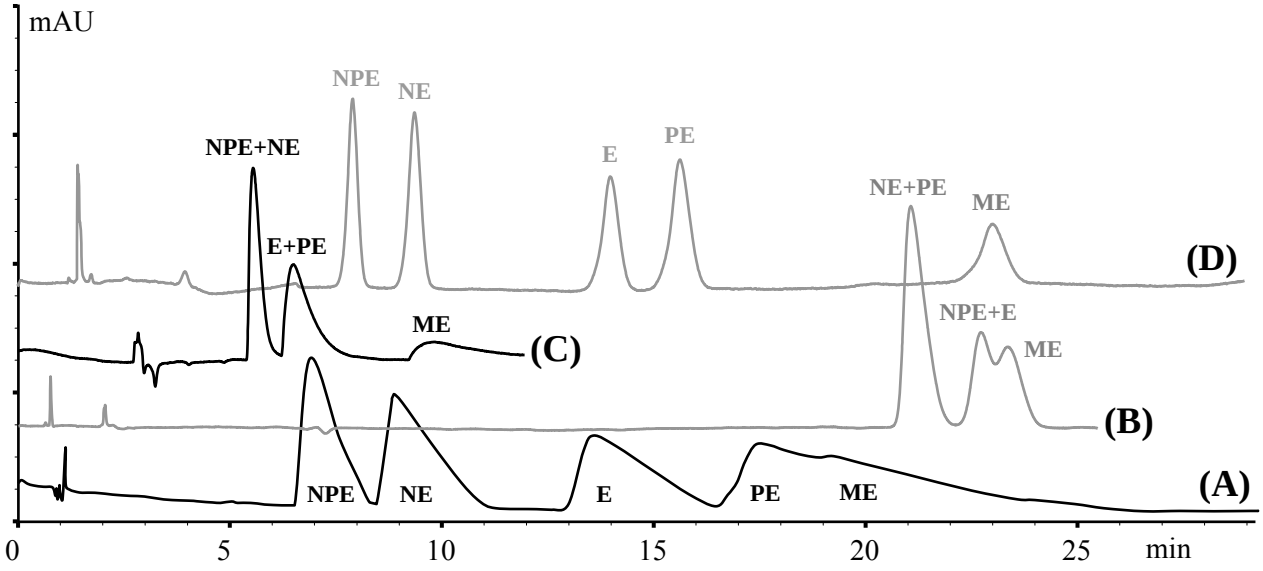


Fig. 3 – Separation of ephedrines on octadecyl silicagel stationary phases. **(A) Column A**/ mobile phase was an aqueous 0.1% (v/v) solution of phosphoric acid at pH=3; **(B) Column A**/ mobile phase was a mixture of methanol and an aqueous 15 mM solution of sodium hexane sulphonate at pH=3 with phosphoric acid in the volumetric ratio 3/7; **(C) Column C**/ mobile phase was a mixture of methanol and an aqueous 15 mM ammonium acetate/acetic acid buffer at pH=3.5 in the volumetric ratio 3/7; **(D) Column A**/ mobile phase was a mixture of methanol and an aqueous 15 mM butylmethylimmidazolium triflate at pH=3.5 with phosphoric acid in the volumetric ratio 3/7.

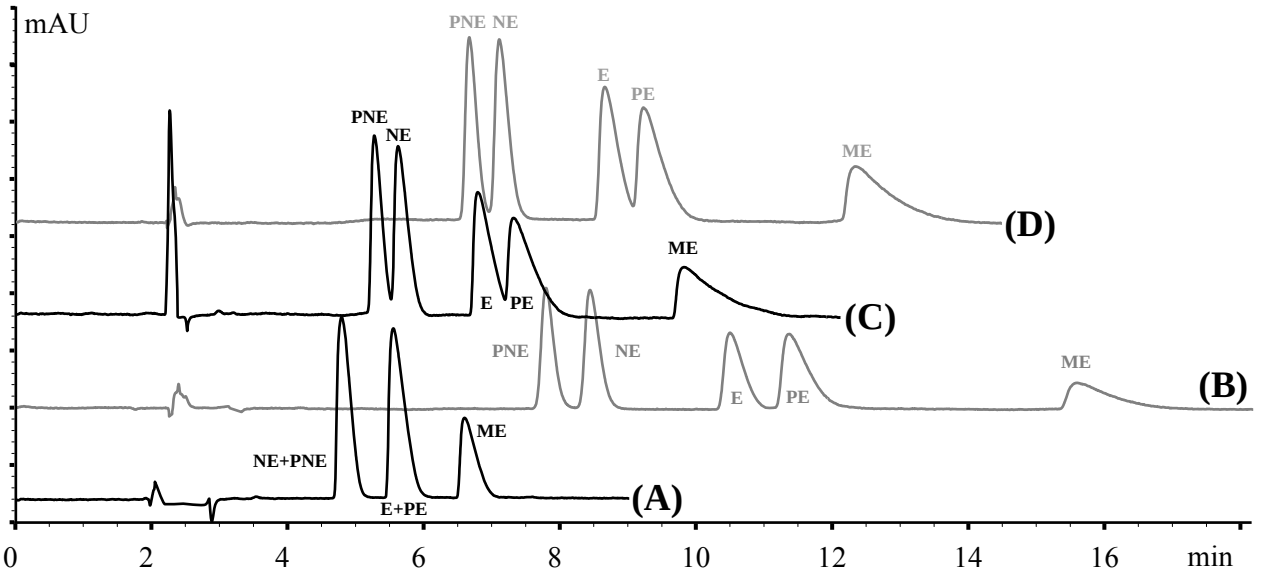


Fig. 4 – Separation of ephedrines on propionitrile silicagel stationary phases. **(A) Column B**/ mobile phase was a mixture of acetonitrile and an aqueous 10 mM solution of ammonium acetate at pH=3.5 with acetic acid in the volumetric ratio 2.5/7.5; **(B) Column B**/ mobile phase was a mixture of methanol and an aqueous 15 mM HFBA acid in the volumetric ratio 17/83; **(C) Column B**/ mobile phase was a mixture of methanol and an aqueous 1% (v/v) acetic acid solution in the volumetric ratio 2/8; **(D) Column B**/ mobile phase was a mixture of methanol and an aqueous 15 mM sodium pentane sulphonate at pH=3.5 with phosphoric acid in the volumetric ratio 2/8.

The use of phenyl chemically modified silicagel as a stationary phase for ephedrines separation was also considered as an experimental alternative. As illustrated in Fig. 5A, the π-π interactions modulating the basic RP separation mechanism lead to an almost baseline separation of the five analytes. Peak tailing still exists, but may be considered as acceptable. If a perfluorinated ion pairing agent is added to the mobile phase (see Fig. 5B), selectivity considerably increases as peak symmetry increases, too.

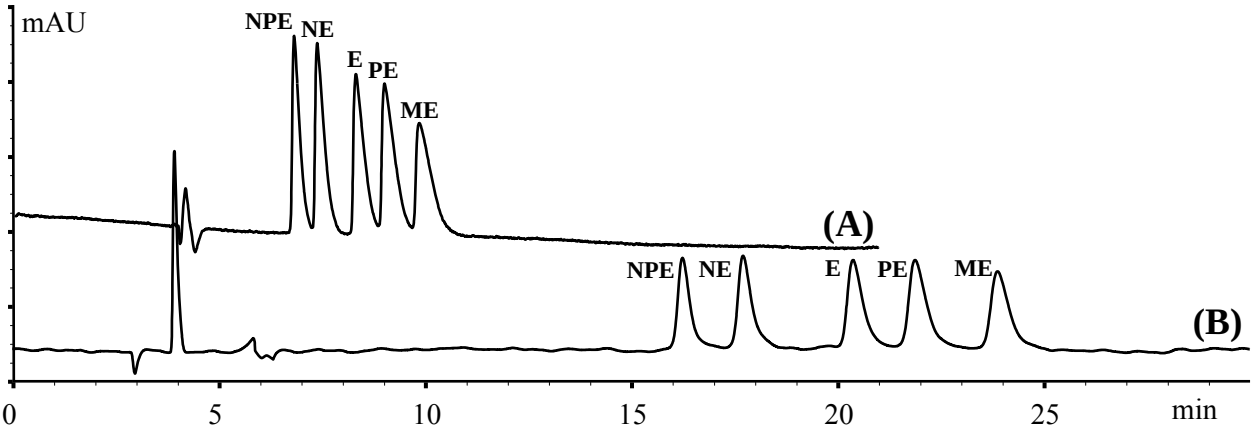


Fig. 5 – Separation of ephedrines on phenyl silicagel stationary phases. **(A) Column D**/ mobile phase was a mixture of acetonitrile and an aqueous 0.1% (v/v) formic acid solution in the volumetric ratio 22/78; **(B) Column D**/ mobile phase was a mixture of methanol and an aqueous 15 mM HFBA solution in the volumetric ratio 28/72.

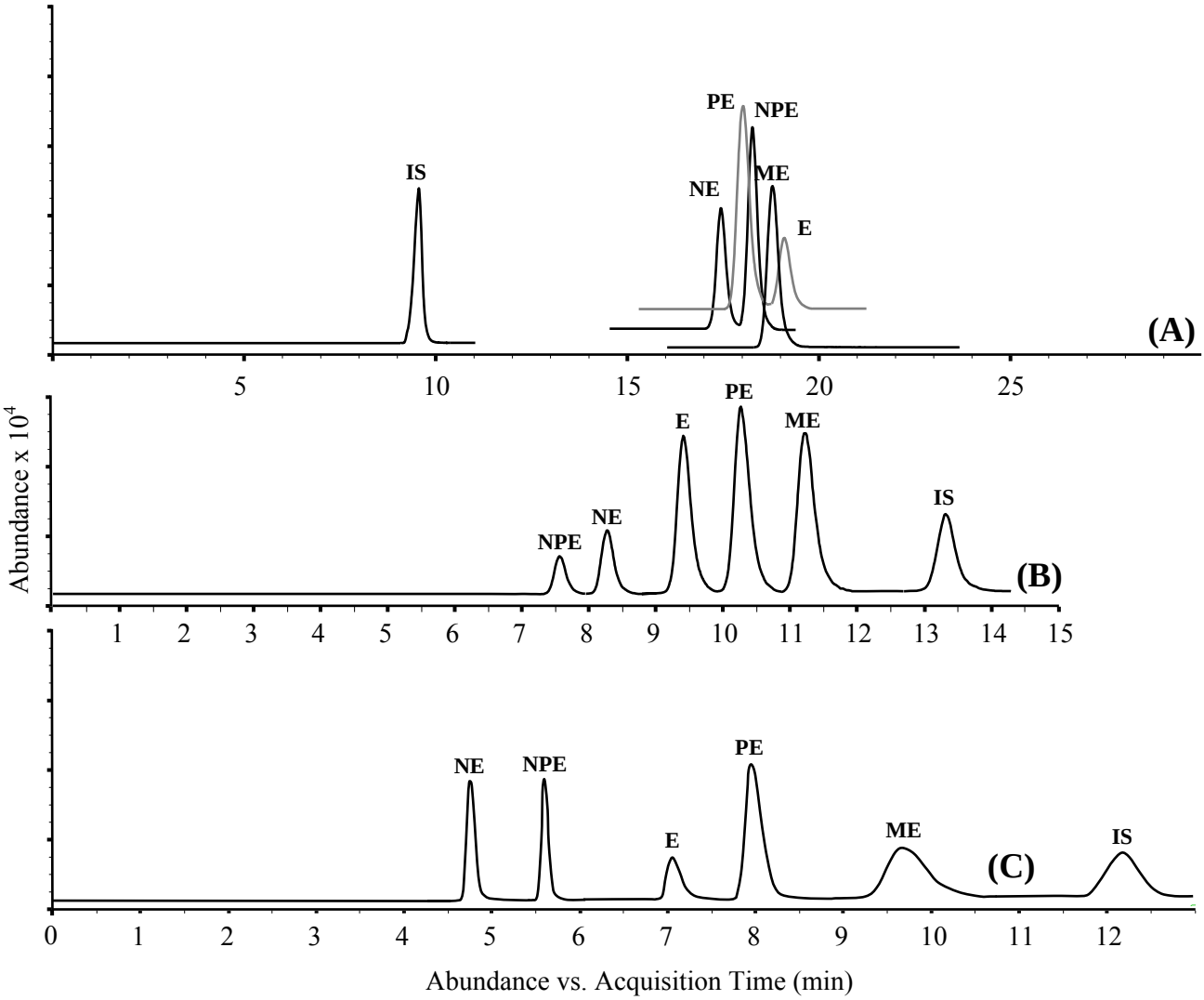


Fig. 6 – Separation of ephedrines through **Method 1** (A), **Method 2** (B) and **Method 3** (C), as described under the Experimental section (*3. Chromatographic conditions*).

From observations being made during method development, we decided to further optimise the topic in three directions, as illustrated in Fig. 6. The first direction consisted in the application of a perfluorinated IP separation mechanism on a highly end-capped octadecyl chemically modified silicagel stationary phase leading to the separation in Fig. 6A. The critical pairs NE/NPE and E/PE are chromatographically baseline solved. However, group separation (according to methyl substitution of the amino group) was not entirely achieved, forcing the use of the additional discrimination selectivity brought by the tandem MS detection system. The resulting peak symmetry is fair, and the detailed experimental conditions are given under the Experimental section, and will be further referred as **Method 1**.

The second direction focussed on the separation on the phenyl chemically modified silicagel. We increased selectivity by shifting from acetonitrile to methanol as organic modifier in the mobile phase. We kept the RP mechanism modulated through the $\pi-\pi$ interactions in a pure form, not altered by ion pairing phenomena. A typical separation is illustrated in Fig. 6B, and experimental conditions are detailed in the Experimental section and will be further addressed as **Method 2**.

Taking into account that octadecyl modified silicagels with polymeric coverage and highly end-capping characteristics are commercially available on the market, and exhibits ability to work in a pH range that exceed the pK_a of the target analytes (more precisely a pH of 10.5), we decided also to explore such direction, as illustrated in Fig. 6C. Detailed separation conditions are given in the Experimental section, and are further addressed in text as **Method 3**. One can observe good selectivity between the considered compounds, and acceptable peak symmetry. Last eluting compounds, namely ME and IS, are separated however, with a lower efficiency (due probably to a slow desorption kinetic from the polymeric adsorbent in the SPE step, making re-focusing less effective).

2. Validation issues

The three optimized methods were submitted to validation. Some of the validation parameters, describing the quality attributes of the analytical methods, are illustrated in Table 1.

The first two methods behave similarly if considering the quantitation limits and the linearity ranges. The third one is more sensitive, which is obvious, as long as about 40 times more absolute amounts of the target compounds are loaded on the analytical column. From the practical point of view, such gain in sensitivity is not absolutely necessary. For instance, in the forensic field, and more precisely for antidoping purposes, only thresholds in the low μg/mL are required. It is worthwhile to note that in the specific conditions of **Method 1**, the accumulation of the perfluorinated ion pair reagent in the ion source leads to a continuous decreasing trend in peak area values (as illustrated in Fig. 7). Method's accuracy is not affected, due to the fact that the IS trend follows the trends of the analytes. This feature will further limit the length of an analytical sequence (at an upper limit of 50 successive injections), in order to fit in the admissible precision (15%) and accuracy (±15%) intervals. Obviously, for **Method 2**, such a decreasing trend in the detector response is not observable, only the acidic additive (formic acid) reaching the ion source (see also Fig. 7).

Another interesting feature refers to the regression model applied for the pair NE/NPE in the conditions of **Method 3**. From Table 1 it clearly results that the regression model was binomial instead of linear (as for **Methods 1** and **2**). This should be related to specific ionization patterns in the source, when the effluent from the analytical column is buffered at an alkaline pH, although ionization still occurs in the positive mode. It is worthwhile to note that NE/NPE fragmentation in the ion source readily occurs in the positive mode, and the molecular ion cannot be isolated. The precursor ion derives from the molecular ion, after the elimination of a water molecule.

When the response domain increases, as for **Method 3**, it becomes mandatory to use $1/x^2$ weighted linear regression model.

The following compounds were studied for potential interferences with the target analytes during their assays with respect to **Methods 1-3**: amphetamine, 6-OH bromantane, bupropione, caffeine, clobenzorex, crotetamide, fencamfamine, phendimetrazine, phenethylline, fenfluramine, phentermine, heptaminol, mephentermine, methamphetamine, methadone, 3,4-MDA, methoxyphenamine, methylphenidate, n-ethylamphetamine, N,N-dimethylamphetamine, pentazocine, pentetrazole, pethidine and prolintane. None of the compounds above listed interfered with the studied analytes following analytical procedures described under **Methods 1-3**.

Table 1

Quality parameters determined during validation of the selected analytical methods for the assay of ephedrines in urine (results are presented in columns in the order NE/NPE/E/PE/ME)

Quality parameter	Method 1	Method 2	Method 3
Limit of quantitation (LOQ) - µg/mL	0.5/0.5/1.0/2.5/1.0	0.25/0.25/0.5/1.25/0.5	0.015/0.015/0.025/0.075/0.025
Upper limit of quantitation (ULOQ) - µg/mL	6/6/12/30/12	5/5/10/15/10	1/1/2/5/2
Regression model	lin.*/lin./ lin./ lin./ lin.	lin./lin./ lin./ lin./ lin.	bin.**/bin./lin($1/x^2$)***/ lin($1/x^2$)/ lin($1/x^2$)
R^2 for linear regression equations	0.9921/0.9971/0.9979/0.9969/0.9983	0.9967/0.9989/0.9983/0.9975/0.9991	0.9917/0.9932/0.9956/0.9968/0.9956
Low QC concentration level- µg/mL	1.0/1.0/2.0/5.0/2.0	0.5/0.5/1.0/2.5/1.0	0.03/0.03/0.05/0.15/0.05
Medium QC concentration level- µg/mL	5.0/5.0/10.0/25.0/10.0	2.5/2.5/5.0/10.0/5.0	0.50/0.50/1.00/2.50/1.00
High QC concentration level- µg/mL	5.3/5.3/10.6/26.5/10.6	3.0/3.0/7.5/12.5/7.5	0.75/0.75/1.50/4.00/1.50
Minimum intraday precision (RSD%)	1.20/1.79/1.33/1.68/1.65	1.10/1.63/1.32/1.72/1.80	2.20/1.96/1.84/2.26/2.27
Maximum intraday precision (RSD%)	2.12/2.64/1.73/1.80/2.37	2.31/2.24/1.96/2.43/2.87	3.51/2.84/2.73/4.33/4.65
Minimum interday precision (RSD%)	0.83/0.52/0.76/0.42/0.57	0.92/0.62/0.95/0.52/0.71	1.75/2.23/1.56/2.36/1.80
Maximum interday precision (RSD%)	6.22/5.61/2.56/2.68/2.33	5.83/4.86/3.21/3.68/4.32	7.91/8.32/5.62/6.84/5.93
Minimum interday accuracy (% bias)	95.3/97.6/98.7/97.4/99.9	98.9/102.8/101.7/101.6/102.9	92.3/95.1/91.8/92.7/97.3
Maximum interday accuracy (% bias)	101.4/105.4/105.4/106.8/106.2	112.1/113.5/112.2/113.9/109.8	107.5/108.9/103.6/111.2/109.5
Matrix effects (mean response ratios for urine/water matrices spiked with analytes)	0.96/1.02/0.97/0.96/1.04	0.98/1.04/1.03/0.98/1.01	1.02/0.98/0.96/1.03/1.06
Selectivity (mean residual peak area in 6 blank urine samples as % from peak area at LLOQ)	0.05/0.12/0.21/0.50/0.35	0.41/0.12/0.06/0.14/0.18	0.32/0.16/0.12/0.31/0.45

* linear regression model; ** binomial regression model; *** $1/x^2$ weighted linear regression model.

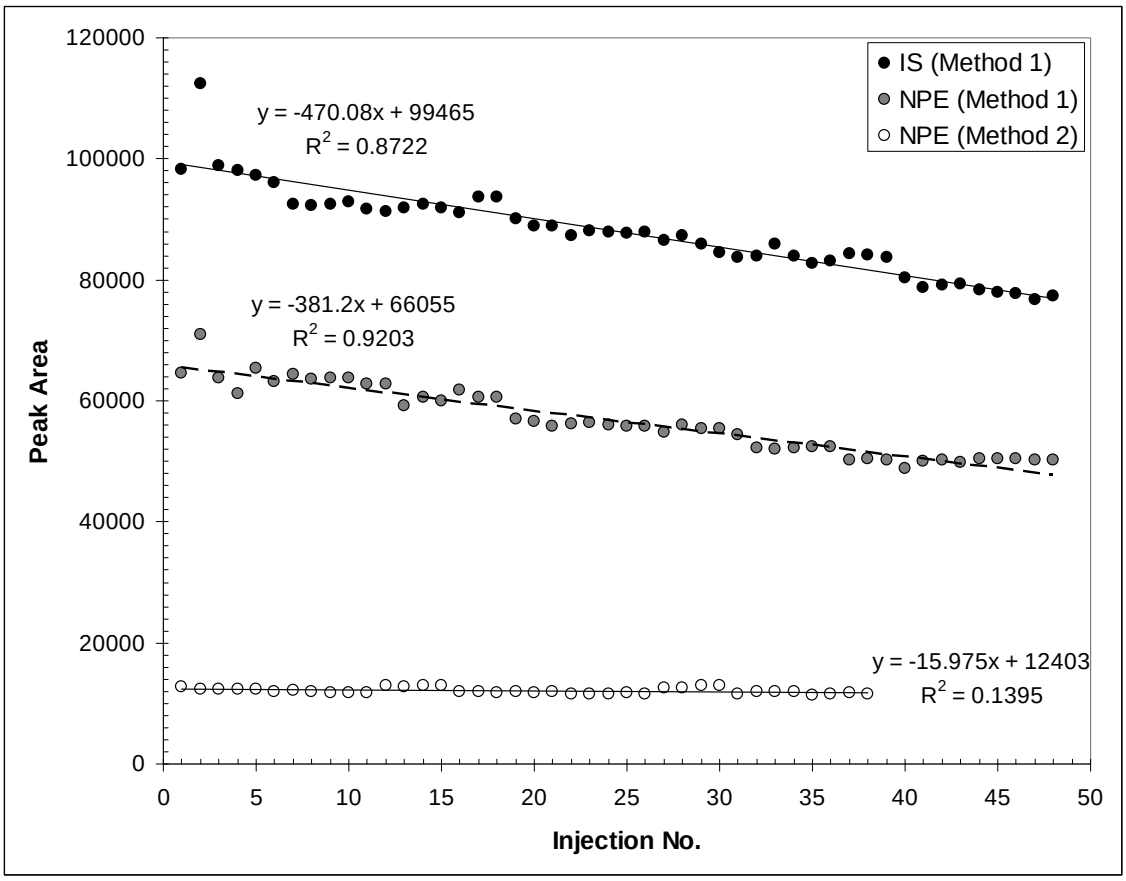


Fig. 7 – Reduction of the ionization yield in the ion source due to the accumulation of the perfluorinated IP reagent, in the conditions of **Method 1**. For **Method 2**, such a negative trend is not observable.

3. Incurred samples, method's comparison

Fifty incurred urine samples were kindly offered by the Laboratory for Doping Control (Romanian Anti-Doping Agency) to be analyzed for ephedrines content. For E, only 21 samples produced results higher than the determined LOQ for **Methods 1** and **2**. These samples were analyzed by means of the validated methods and the results were compared by means of the Bland-Altman approach with tolerance intervals.[32] The comparisons are presented in Fig. 8. One can observe that the three methods behave similarly. Results are in fair agreement even for samples having determined concentration values significantly higher than the determined upper limit of quantitation. Through comparing M1 to M2 and M1 to M3, respectively, only one pair of results was placed outside the accepted interval.

CONCLUSIONS

Although simple with respect to their chemical structures, ephedrines require special chromatographic elution conditions in order to simultaneously preserve selectivity against the critical pairs NE/NPE and E/PE and peak symmetry. After studying the retention behaviour on different stationary phases and separation mechanism, three alternatives were retained: one uses IP retention mechanism with a perfluorinated ion pairing agent (HFBA) on an octadecyl chemically modified silicagel, the other uses an RP retention mechanism modulated by means of the π-π interactions on a phenyl modified silicagel, while the last one requires alkaline elution conditions under RP separation mechanism on polymeric, strongly end-capped octadecyl silicagel. The last method used an on-line SPE/RPLC approach, the SPE procedure being achieved on a vinylpyrrolidone/divinylbenzene copolymer (Oasis HLB).

The proposed methods were validated, and behave similarly with respect to most of their quality characteristics. Incurred urine samples being analyzed by means of the three alternatives produces comparable results. Comparison between the concentration values obtained for ephedrine (E) by means of the Bland-Altman approach revealed the good correlation between the results produced

through application of the three alternative methods on the incurred samples, although some of the experimental values exceeded the upper limit of quantitation (ULOQ) determined during validation.

Acknowledgements: Authors acknowledge the financial support given by the Roumanian project PNII_ID_PCE_2011_3_0152.

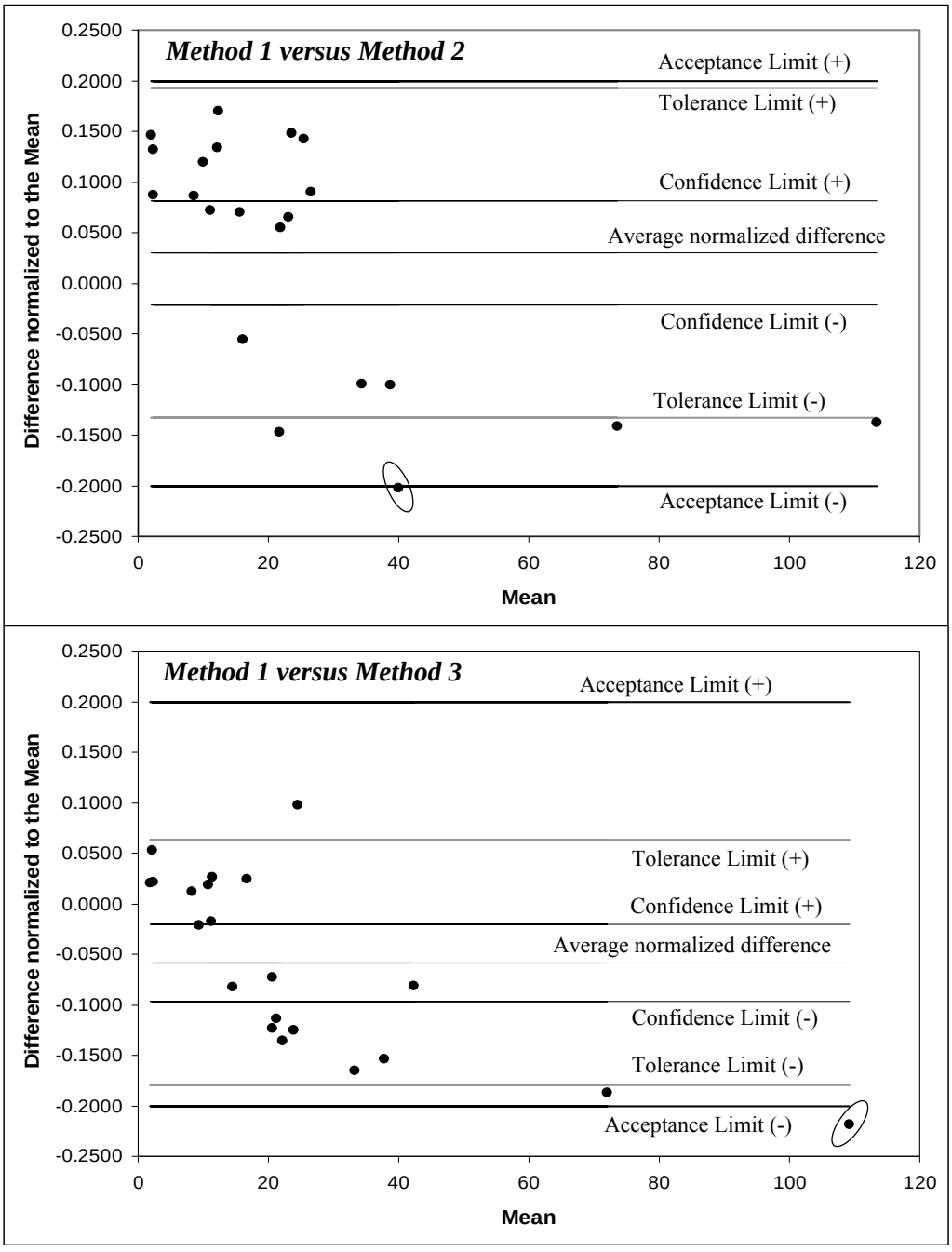


Fig. 8 – Bland-Altman plots for comparison of **Method 1** to **Method 2** and **3** respectively, illustrating the reciprocal good fitting between experimental results referring to the analyte Ephedrine (E) in incurred urine samples.

REFERENCES

1. F. Pellati and S. Benvenuti, *J. Pharm. Biomed. Anal.*, **2008**, *48*, 254-263.
2. M.Y. Heng, S.N. Tan, J.W.H. Yong and E.S. Ong, *TrAC*, **2013**, *50*, 1-10.
3. S. Strano-Rossi, S. Odoardi, E. Castrignano, G. Serpelloni and M. Chiarotti, *J. Pharm. Biomed. Anal.*, **2015**, *106*, 144-152.
4. K. Oekusa, T. Ono, M. Hayashida, M. Nihira and Y. Ohno, *Legal Medicine*, **2009**, *11*, S573-S575.
5. Y. Shi, C. Sun, B. Gao and A. Sun, *J. Chromatogr. A*, **2011**, *1218*, 7655-7662.

6. M.H. Spyridaki, P. Kiousi, A. Vonaparti, P. Valavani, V. Zonaras, M. Zahariou, E. Sianos, G. Tsoupras and G. Georgakopoulos, *Anal. Chim. Acta*, **2006**, *573-574*, 242-249.
7. T. Baciu, F. Borrull, C. Aguilar and M. Calull, *Anal. Chim. Acta*, **2015**, *856*, 1-26.
8. F. Badoud, E. Grata, L. Perrenoud, M. Saugy, S. Rudaz and J.L. Veuthey, *J. Chromatogr. A*, **2010**, *1217*, 4109-4119.
9. K. Deventer, O.J. Pozo, P. Van Eenoo and F.T. Delbeke, *J. Chromatogr. B*, **2009**, *877*, 369-374.
10. H. Fang, M. Liu and Z. Zeng, *Talanta*, **2006**, *68*, 979-986.
11. J.M. Plotka, M. Biziuk, C. Morisson and J. Namiesnik, *TrAC*, **2014**, *56*, 74-89.
12. A.A. Younes, H. Ates, D. Mangelings and Y. Vander Heyden, *J. Pharm. Biomed. Anal.*, **2013**, *75*, 74-85.
13. C.P. Kapnissi-Christodoulou, I.J. Stavrou and M.C. Mavroudi, *J. Chromatogr. A*, **2014**, *1363*, 2-10.
14. J.M. Pallicer, J. Sales, M. Roses, C. Rafols and E. Bosch**,** *J. Chromatogr. A*, **2011**, *1218*, 6356-6368.
15. D.S. Bell, H.M. Cramer and A.D. Jones, *J. Chromatogr. A*, **2005**, *1095*, 113-118.
16. M. Kagan, M. Chlenov, S. Melnikov, A. Greenfield, J. Gross and R.C. Bernotas, *J. Chromatogr. A*, **2008**, *1194*, 80-89.
17. Z. Aturki, G. D'Orazio, A. Rocco, K. Si-Ahmed and S. Fanali, *Anal. Chim. Acta*, **2011**, *685*, 103-110.
18. J. Heaton, N. Gray, D.A. Cowan, R.S. Plumb, C. Legido-Quigley and N.W. Smith, *J. Chromatogr. A*, **2012**, *1228*, 329-337.
19. F. Wei, M. Zhang and I.Q. Feng, *J. Chromatogr. B*, **2007**, *850*, 38-44.
20. P.W. Stege, A.V. Lapierre, L.D. Martinez, G.A. Messina and L.L. Sombra, *Talanta*, **2011**, *86*, 278-283.
21. W. Wang, C. Li, Y. Li, Z. Hu and X. Chen, *J. Chromatogr. A*, **2006**, *1102*, 273-279.
22. L. Zhou, X. Zhou, Z. Luo, W. Wang, N. Yan and Z. Hu, *J. Chromatogr. A*, **2008**, *1190*, 383-389.
23. J. Zhou and Z. Zeng, *Anal. Chim Acta*, **2006**, *556*, 400-406.
24. A.J. Midey, A. Patel, C. Moraff, C.A. Krueger and C. Wu, *Talanta*, **2013**, *116*, 77-83.
25. S. Schneiders, T. Holdermann and R. Dahlenburg, *Sci. & Jus.*, **2009**, *49*, 94-101.
26. I.K. Dijiba, A. Zhang and T.M. Niemczyk, *Int. J. Pharm.*, **2005**, *289*, 39-49.
27. S.S.M. Hassan, A.H. Kamel and H. Abd El-Nabi, *Talanta*, **2013**, *103*, 330-336.
28. C.K. Zacharis and P.D. Tzanavaras, *Anal. Chim. Acta*, **2013**, *798*, 1-24.
29. L. Fotouhi, Y. Yamini, S. Molaei and S. Seidi, *J. Chromatogr. A*, **2011**, *1218*, 8581-8586.
30. D.L. Deng, J.Y. Zhang, C. Chen, X.L. Hou, Y.Y. Su and L. Wu, *J. Chromatogr. A*, **2012**, *1219*, 195-200.
31. L. He, W. Zhang, L. Zhao, X. Liu and S. Jiang, *J. Chromatogr. A*, **2003**, *1007*, 39-45.
32. F.E. Lytle, R.K. Julian and A.M. Tabert, *Bioanalysis*, **2009**, *1*, 705-714.